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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Levenberg <i>et al.</i>	Examiner:	Sgagias, M.K.
Serial Number:	10/731,672	Art Unit:	1632
Filed:	December 9, 2003		
For:	ENGINEERING THREE-DIMENSIONAL TISSUE STRUCTURES USING DIFFERENTIATING EMBRYONIC STEM CELLS		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Declaration under 37 C.F.R. § 1.131

I, Shulamit Levenberg, Ph.D., declare as follows:

1. I am currently an Associate Professor in Biomedical Engineering Department at Technion, Israel Institute of Technology, Israel. I received my Ph.D. from Weizmann Institute of Science, Rehovot, Israel in Molecular Cell Biology in 1999, and worked in Prof. Robert Langer Lab at Massachusetts Institute of Technology, U.S.A., from 1999 to 2004.
2. I am an inventor of the subject matter disclosed and claimed in the present application, U.S.S.N. 10/731,672, filed December 9, 2003, and entitled "ENGINEERING THREE-DIMENSIONAL TISSUE STRUCTURES USING DIFFERENTIATING EMBRYONIC STEM CELLS" (the '672 application). This application claims priority to U.S. Provisional Patent Application Serial No. 60/432,228, filed on December 10, 2002 (the '228 application), and U.S. Provisional Patent Application Serial No. 60/443,926, filed on January 31, 2003 (the '926 application).
3. This declaration is presented for the purpose of removing from consideration by the Examiner the reference by Griffith, *et al.* (Science, 295: 1009-1014, 2002) (hereafter "Griffith"). This article bears a publication date of February 8, 2002. Therefore, Griffith became available to

the public less than one year prior to the filings of the '228 application and the '926 application, to which the present application claims priority.

4. The present Declaration is presented in accordance with *In re Stompel*, 113 U.S.P.Q. 77 (CCPA 1957) and establishes conception and reduction to practice of the invention in this country before February 8, 2002, the effective date of the cited Griffith.

5. On a date before February 8, 2002, I, together with co-inventors Ngan F. Huang, Erin B. Lavik, Joseph Itskovitz-eldor, and Robert Langer conceived and reduced to practice tissue engineering constructs comprising embryonic stem cells, a three-dimensional cell support polymer matrix, and at least one growth factor selected to promote differentiation of the stem cells, and methods thereof.

6. **Exhibit A** is a copy of two pages from my laboratory notebook, with dates blacked out. Exhibit A provides evidence of conception and actual reduction to practice of tissue engineering constructs comprising embryonic stem cells, a porous three-dimensional cell support polymer matrix, and at least one growth factor selected to promote differentiation of the stem cells, and methods thereof prior to February 8, 2002.

7. **Exhibit A** includes a description of experiments in which exemplary growth factors were used to differentiate stem cells on polymer scaffolds into tissue-like structures. The first page shows the details of preparation of polymer scaffolds and stem cells from embryoid bodies (EBs) with different growth factors. The second page shows results of seeding stem cells onto the polymer scaffolds with growth factors. For example, retionic acid (RA), a growth factor, was added to form neural tissues. The dates on which the experiments were performed are found on the notebook, and are prior to February 8, 2002.

8. The laboratory notebook pages were prepared in the United States of America.

9. I declare that all statements made herein of my own knowledge are true, and that those statements made on information and belief are believed to be true; and further that these

statements were made with the knowledge that willful, false statements and the like are made punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful, false statements may jeopardize the validity of the '542 application or any patents that may issue thereon.

Shulamit Levenberg

Shulamit Levenberg, Ph.D.

____6.July .2010____
Date

EXHIBIT A

EB on Scaffold Expt: Comparison of RA, TGFB, and Actavin Growth Factors

METHODS:

Preparation of Scaffolds:

1. Sterilized 10 polymer scaffolds overnight in 70% ETOH.
2. Soaked scaffolds in 3 changes of PBS, about 5 mins each change. Transferred with sterilized forceps.

Preparation of EBs:

3. Transfer EBs in media to 50 mL Falcon tube. Pipetted lightly to remove EBs adhered to plate. Washed plate with 5 mL old media.
4. Removed most supernatant. Resuspended in 5 mL new EB media. Transferred to 15 mL Falcon tube.
5. Centrifuged at 800 for 1 minute with brake.
6. Diluted mef trypsin 1:5 (white: 25g porcine tryp/L in 0.9% NaCl) in PBS. Add 2 mL diluted trypsin to the 10mL EB/media mixture. Resuspended by pipetting.
7. Incubated for 5 mins. Added 4mL TNS to cells. Resuspended, and centrifuged at 800 for 3 min. Remove supernatant. Add EB media to aliquot the cells into 2:1 (for scaffolds/EB: EB samples)

Scaffold Conditions:

GF	Stock	Working	# scaffolds	EB Plates w/o Scaffold
RA	7.5mg/mL in DMSO	(1:25000)	2	1
TGF-B	2ug/mL	(1:1000)	2	1
Activin	20ug/mL	(1:1000)	2	1
Activin & TGF-B		(1:1000)	2	1
Normal media			2	1

8. Prepared 25mL EB media with GFs. (RA 1:25000; TGF-B 1:1000; Act 1:1000)
9. Added 1mL of respective media to each well to be used for scaffolds. Added scaffolds to wells.
10. Prepared matrigel 1:1 with respective media (50 uL matrigel with each media).
11. Aliquotted cells into eppendorfs. Spin down <1000 for 4 min. Remove supernatant carefully. Add 20-25 uL matrigel-media to cells. Mix well.
12. Removed media from scaffolds in wells.
13. Added 20-25 uL cell-matrigel mixture onto each scaffold.
14. Incubate 30 mins to solidify matrigel.
15. Added 4 mL respective media to each well.
16. For remaining EB's aliquot, spin down and add media. Pipette into wells and add respective media.
17. Placed on shaker.

10 EB on Scaffold with GF Expt

Scaffold - PLGA

70%

1. Sterilize polyim. scaffold in EtOH overnight
2. Rinse in PBS: Pour out until only 1.0ml EtOH Rem PBS in small wells. Pour all scaffold EtOH into plate. Transfer into PBS in 6-well plates with forceps.
3. Transfer 24 for ~5min each

EB (pyrolytic)

4. Transfer EB media to 50ml Falcon. Pipette lightly to remove EBs stuck to plate. Wash with clean media or old media.
5. Remove supernatant water. Smt left. Resuspend in new media (50ml). Transfer to 15ml Falcon. Use same Smt for all 5 tubes.
6. Repeat again with Smt S. Centrifuge 800 for 1min with brake.
7. Dilute Trypsin (Gibco) (10X): 1 → 5. Add 2ml diluted to the 10ml EB-media mixture. Resuspend by pipetting. Pour into 2 small petri dishes (5ml each).
8. Incubate 3min in the 15ml Falcon

[Dilute Trypsin 1:5 using PBS → only for EB's]

Scaffold Conditions - 10

- | | |
|-------------------|---|
| 1. RA | 2 |
| 2. TGFβ | 2 |
| 3. Activin | 2 |
| 4. TGFβ + Activin | 2 |
| 5. control | 2 |

EB's w/o scaffold

- | |
|----------------|
| TGFβ |
| activin |
| RA |
| TGFβ + activin |
| ⊖ : EB media |

(RA: neuronal, Activin + TGF = muscle)

9. Add 25ml EB media in 50ml Falcon. Add GF's

RA 25ml (should be 1ml/25ml)
TGFβ 1ml per 1ml media
Activin 1ml per 1ml media

10. Use 6-well plates "not from tissue culture"
11. Add 4ml TBS to cells. Resuspend. Centrifuge at 800, 3min
12. Prepare matrigel 1:1 with the right media. 50ml of each.

[Cell Counting: use eastern pipette. Count # cells in 5x5 area. Each square has 6 sq.]

$$\# \text{ cells} \times 10^4 / \text{ml} =$$

$$\text{counted } 200 \times 10^4 = 2 \times 10^6 / \text{ml cells} \times 6 \text{ ml} = 12 \text{ million cells}$$

13. Aliquot the cells. Spin down. Add the matrigel media into the cells. Resuspend.